WHEY PRESERVATION BY HYDROGEN PEROXIDE

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SUMMARY

Concentrations of 0.02% H_2O_2 in cheese whey exerted a preservative effect for as long as ten days. Bacterial plate counts were made of peroxide-preserved wheys of pH 4.9, 5.9, and 6.9 kept at 25, 37, and 50° C. According to these data, H_2O_2 addition shortly after separation of the whey from curd is recommended. Peroxide addition to grossly contaminated wheys of 2.8×10^7 microorganisms per milliliter resulted in a 97% bacterial kill within 1 hr. The 0.02% H_2O_2 concentration was relatively ineffective against greater numbers of bacteria. *Saccharomyces fragilis* yeast was propagated in peroxide-preserved whey from which the peroxide had been removed by the action of catalase. Results were comparable to a control fermentation on untreated fresh whey.

Enormous quantities of liquid whey from cheese are available for utilization. The United States cheese-making industry alone produces over twelve billion pounds of whey annually (8). Although it is economically impractical to consider utilization of this entire amount, increased uses for a greater proportion of the whey are possible. Suggested outlets are many (3), ranging from returning liquid whey to the farms for livestock feeding to using it as a supplemented medium in the fermentation industry.

Liquid whey remaining from the cheese-making process is composed of about 4 to 5% lactose and 0.9% protein, and constitutes a good bacterial substrate. Bacterial decomposition of this whey is a problem when storage or transportation is necessary. The use of a relatively harmless preservative may be advisable, especially if the whey is to be used for microbiological processes. In addition to being nontoxic, the preservative should be inexpensive, nonreactive with whey components, and be easily destroyed or removed when necessary.

Hydrogen peroxide has long been recommended as a dairy preservative in tropical countries. Excellent comprehensive reviews on the subject have been prepared by Rosell (10) and Lück (5). This strong and active germicide has been found acceptable in preventing souring of milk during transport and in preserving milk for cheese-making. In H_2O_2 milk-preservation methods practiced abroad, an addition of catalase is often made before the milk is ready for human consumption. This enzyme has the property of splitting H_2O_2 into its breakdown products, water and oxygen. Its use has been studied by Curran, Evans, and Leviton (2) in sporicidal action of hydrogen peroxide.

The widespread and sanctioned use of H_2O_2 as a milk preservative in some countries (9) suggested laboratory investigations into its applicability as a preservative for liquid whey. Previous studies in this area have been made by Plöttner (7), who used 0.015 to 0.03% H_2O_2 (by weight) in the preservation of

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raw sweet whey and removed H_2O_2 residues by heat precipitation of the proteins or by addition of yeast. Results of our laboratory investigations on the peroxide-preservation of whey and its subsequent use for yeast growth are given in this report.

MATERIALS USED

The laboratory studies were made on liquid whey obtained from a local cottage cheese–manufacturing concern. The whey as it was received from the plant had a pH of 4.5 and temperature up to 60° C. It was transported to the laboratory in milk cans and placed in a refrigerated room at 4° C. immediately upon arrival. The insoluble proteins settled readily, and only the supernatant fluid was used for these studies.

Reagent-grade hydrogen peroxide of 28–30% purity was employed as the preservative, but data are reported on the basis of 100% purity H_2O_2. Exact composition was determined and frequently checked by Kingzett's iodine-thiosulfate titration method (11). The H_2O_2 was kept at 4° C. to prevent unnecessary deterioration due to room temperatures.

The inoculum used for testing preservation action was propagated in the laboratory at room temperature throughout this series of experiments. Sporadic feedings of both dried skimmilk and liquid whey were made to maintain its vigor. The culture consisted of an aerated sludge mixed with commercial milk that had soured. No attempt was made to identify the organisms in the mixture, except for testing their ability to attack milk and whey. An earlier investigation and isolation of the aerated sludge organisms, however, showed the predominance of *Alcaligenes, Flavobacterium, Micrococcus, Pseudomonas, Bacillus,* and *Bacterium* genera (4).

Hydrogen peroxide destruction was catalyzed by solutions of a commercial catalase preparation, Armalase A-100, a purified preparation of the enzyme manufactured by Armour Company.[2] Its potency is 100 Keil units per milliliter, one unit being the quantity needed to decompose 1 g. of 100% H_2O_2 in 10 min. at 25° C., in an inert atmosphere of CO_2 or N_2 (1).

STUDIES ON MINIMUM EFFECTIVE H_2O_2 CONCENTRATIONS

Preliminary studies were made on 50-ml. quantities of whey placed in sterile Erlenmeyer flasks having cotton plugs. Varying protective amounts of H_2O_2 were added. Half of the flasks were inoculated with the test organisms (50,000 organisms per flask); the remaining flasks were to contain only indigenous whey organisms. The flasks were held at room temperature and examined daily for physical changes in appearances. Bacterial growth was detected by transferring a loopful of the whey to sterile whey-nutrient broth solution and observing turbidity after incubation at 30° C. Results of these tests are given in Table 1 and point to 0.020% H_2O_2 as the effective level of protection. For ten days of the experiment, the broth tubes gave like turbidity readings, showing that the peroxide

[2] It is not implied that the USDA recommends the above company or its product to the possible exclusion of others in the same business.

TABLE 1

Bactericidal effect of H_2O_2 as determined by a sterility test procedure

	Age of wheys (in days)					
	1–3		4–7		8–10	
	Bacterial growth in sterile broth					
H_2O_2 in whey	I	U	I	U	I	U
(%)						
0	+	+	+	+	+	+
.010	+	−	+	−	+	−
.015	+	−	−	−	+	−
.020	+	−	−	−	−	−
.025	−	−	−	−	−	−
.030	−	−	−	−	−	−

I = Inoculated whey; U = uninoculated whey.

had checked further bacterial growth. On the 11th day, tests for residual H_2O_2 showed traces still present in all wheys. Excess quantities of sterile (Seitz-filtered) catalase were added on the 13th day to destroy the H_2O_2 and to leave the whey susceptible to possible bacterial attack. The next day, the whey solutions were reinoculated with the test organisms and reincubated at 30° C. All the flasks showed bacterial turbidity, proving that the catalase had removed all the H_2O_2.

Table 2 gives results of a test in which 1-liter amounts of whey from the cheese vat were held at room temperature (22–25° C.) in glass flasks covered with beakers. These received no supplementary organisms. Standard bacterial plate counts were made on nutrient agar incubated at 37° C. for 48 hr., as used in water analyses. Only the unprotected whey showed evidence of contamination. After 2 wk., a dense growth of a fungus, presumably *Aspergillus niger,* had appeared on the surface of all three flasks. However, for six days at room temperature, these commercial wheys had been satisfactorily protected from bacterial growth.

TEMPERATURE AND PH EFFECTS ON THE BACTERIAL ACTION OF H_2O_2

The bactericidal action of 0.02% H_2O_2 concentration at different temperatures was investigated. One hundred–milliliter quantities of refrigerated cheese whey were put into sterile bottles and equilibrated at 50, 37, or 25° C. Sufficient bacterial inoculum obtained from well-aerated sludge culture was added to give 1,000 organisms per milliliter of whey. One milliliter of 2% H_2O_2 was added, resulting in a 0.02% concentration in the final whey. At 10-min. intervals,

TABLE 2

Preservation of fresh whey by H_2O_2 as measured by bacterial plate counts[a]

	Bacterial count per milliliter whey		
H_2O_2	First day	Fourth day	Sixth day
(%)			
0	51	2,500	650,000
.020	0	0	0
.025	0	0	0

[a] 48-Hr. growth on nutrient agar at 37° C.

samples were removed for plate count determinations on nutrient agar medium. The bacterial colonies were counted after 48 hr. of incubation at 37° C.

Figure 1 shows the destruction attained at the three temperatures, as plotted on a semilog scale. Most rapid killing was observed at 50° C. Here, the high initial temperature alone had reduced the bacterial count from over 1,000 organisms per milliliter to 230. Within 5 min., 75% of the remaining organisms was destroyed by the H_2O_2. At 37 and 25° C., similar, but not such marked, effects of H_2O_2 were noted. Seventy-five per cent destruction was obtained within 13 min. at 37° C. and within 24 min. at 25° C. At none of these temperatures did the 0.02% H_2O_2 concentration achieve 100% destruction.

Table 3 summarizes the combined effects of temperature, pH, and 0.02% H_2O_2 on the viability of organisms in whey solutions. Natural whey of pH 4.9 and wheys adjusted to pH 5.9 and 6.9 with NaOH were heated to indicated temperatures and inoculated to contain 1,000 organisms per milliliter. After removing the samples for initial bacterial counts, peroxide was added. Samples were removed after 10 and 20 min. for bacterial counts. High temperatures and low pH had an immediate destructive effect, particularly at pH 4.9 and 50° C., destroying over 99% of the original organisms. The 25 remaining colonies were almost all yeasts and fungi, forms somewhat refractory to peroxide treatment as indicated by the 68% kill figure. In spite of the different beginning temperatures, pH's, and variations in viable organisms, maximum destruction occurred in all cases within 10 min. after peroxide addition. Samples taken even after an exposure of 1 hr. to peroxide showed practically no further destruction of the microorganisms.

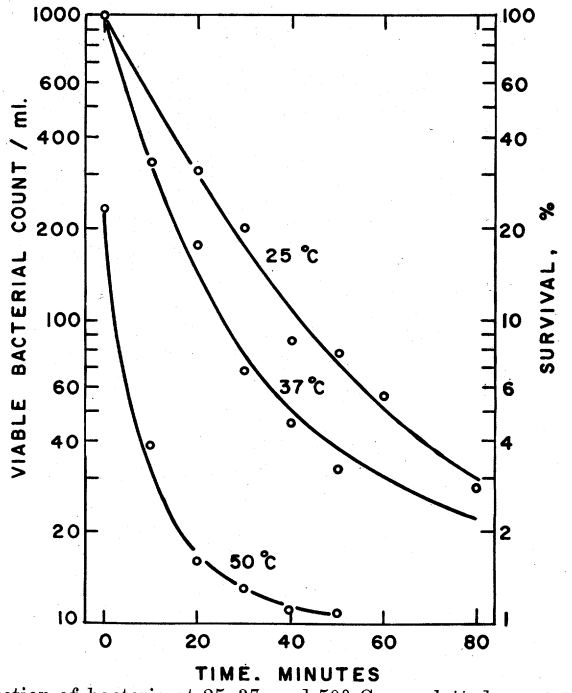

FIG. 1. Destruction of bacteria at 25, 37, and 50° C., as plotted on a semilog scale.

TABLE 3

Bacterial destruction in inoculated whey treated with 0.02% H_2O_2 as affected by temperature and pH

Time	25° C.		37° C.		50° C.	
(min.)	*(No/ml)*[a]	*(%)*	*(No/ml)*[a]	*(%)*	*(No/ml)*[a]	*(%)*
			pH 4.9			
0	537		817		25	
10	35	(93)	24	(97)	8	(68)
20	25	(95)	18	(97)	8	(68)
			pH 5.9			
0	842		931		349	
10	56	(93)	35	(96)	9	(97)
20	34	(96)	32	(96)	6	(98)
			pH 6.9			
0	1,000		1,035		573	
10	181	(82)	61	(94)	8	(98)
20	61	(94)	64	(94)	4	(99)

Figures in parentheses represent percentage of bacteria killed, based on actual zero-hour figures. Percentage kill would be greater if based on initial inoculation of 1,000 organisms per milliliter.

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

BACTERICIDAL POTENCY OF 0.02% H_2O_2

Test organisms were used in these laboratory studies in concentrations of 1,000 organisms per milliliter of whey for these reasons: first, heated wheys received from the cheese manufacturer numbered from zero to about 400 bacteria per milliliter; secondly, 1,000 bacteria per milliliter furnished a convenient number for making bacterial plate counts without further dilution. The destructive effect of 0.02% H_2O_2 on large numbers of bacteria as may occur in contaminated whey remained to be determined. For this work, a bacterial culture was propagated from a contaminated commercial yoghurt preparation and from soured milk, and was considered representative of dairy contaminants. Bacteria numbered 2.8×10^9 in the undiluted culture. Five tenfold dilutions were made in whey. Hydrogen peroxide was added to each flask to give a 0.02% final concentration.

Table 4 presents the viable plate counts and per cent destruction achieved by 0.02% H_2O_2 acting on ranges of bacteria from 10^9 to 10^4. This concentration effected 97% destruction of 2.8×10^7 bacteria after 1 hr. of contact. After 24 hr., a 99% kill was measured. Apparently, the peroxide effect is completed within the 1st hr., as seen also with the other dilutions. The lower percentage of destruc-

TABLE 4

Destruction of bacteria in contaminated whey by 0.02% H_2O_2

Original count[a]	After 1 hr.	Kill	After 24 hr.	Kill
(per ml.)		*(%)*		*(%)*
2.8×10^9	3.6×10^9	0	2.7×10^9	Negligible
2.8×10^8	1.8×10^8	36	2.1×10^8	25
2.8×10^7	0.087×10^7	97	0.023×10^7	99
2.8×10^6	0.0085×10^6	99+	< 10	99+
2.8×10^5	0.00035×10^5	99+	< 10	99+
2.8×10^4	0	100+	< 10	99+

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

tion that occurred with the higher concentration of cells may be explained by the probable presence of catalase, elaborated by greater numbers of catalase-producing organisms. The effect of the small amount of peroxide could be quickly negated, and the bacteria would continue multiplying.

PROPAGATION OF YEAST IN PEROXIDE-PRESERVED WHEY

The effect of peroxide treatment on whey used for subsequent propagation or fermentation was investigated. One liter of liquid whey was treated with 0.02% H_2O_2 and held uncovered at room temperature (24° C.) for 24 hr. Excess catalase was added (30 units of Armalase) and allowed to stand for 30 min. Spot-testing with potassium iodide showed that all the peroxide was gone. This whey was used as part of a growth medium for the propagation of the yeast, *Saccharomyces fragilis* (12). A control fermentation with untreated whey also was run. Data compiled during these fermentations are compared in Table 5. After 3 hr., both fermentations were terminated. The harvest was about the same, and only minor differences were observed between the two fermentations.

DISCUSSION

The 0.02% H_2O_2 concentration showed both a bactericidal and bacteriostatic action against 1,000 bacteria added per milliliter of whey, and inhibited bacterial growth for as long as ten days. Traces of peroxide were easily and completely removed by the enzymatic action of subsequently added catalase, leaving the solution again susceptible to microbial attack. Satisfactory removal was demonstrated also by the successful propagation of yeast in cottage cheese whey preserved with peroxide.

Optimum conditions for peroxide treatment were suggested in the study on temperature and pH effects. Waste whey is available from cottage cheese–making operations at temperatures up to 60° C. at a pH of about 4.5 These conditions are not favorable for bacterial growth. On cooling to only 37° C., however, this whey becomes increasingly attractive to bacteria. Peroxide addition is advisable, therefore, as soon as the whey is collected.

The rate and degree of bacterial destruction by peroxide is dependent on the numbers and types of organisms and on the concentration of peroxide used. The bacterial mixture used in our experiments constituted a stringent test. This culture contained bacteria, yeast, and fungi ordinarily associated with aerobic dairy waste treatment (4). It should be noted that although 0.02% H_2O_2 accomplished up to 98% destruction of these test organisms within as little as 10 min. (at 50° C. and pH 6.7), complete destruction of all growth was

TABLE 5

Propagation of *Saccharomyces fragilis* in fresh and 0.02% H_2O_2-treated wheys

Time	Yeast count		Dry weight		Lactose		pH	
	Fresh	Treated	Fresh	Treated	Fresh	Treated	Fresh	Treated
(hr.)	*(× 10^6/ml)*		*(mg/ml)*		*(mg/ml)*			
0	1,450	2,010	12.96	13.14	39.6	40.2	5.5	5.5
2	3,540	3,460	27.99	28.10	17.6	19.6	5.8	5.95
3	5,380	4,380	36.32	36.04	2.2	3.5	5.2	4.85

not attained. After the rapid initial killing, the peroxide effect seemed to slacken. In other tests with this same mixture of organisms, even 24 hr. of exposure to 0.02% H_2O_2 did not show 100% reduction in bacterial count. Varying degrees of resistance and indifference to the preservative are expected in such a mixture of microorganism. The susceptible species, primarily the Gram-negative forms, are annihilated; others, such as the Gram-positive rods, are unaffected and continue to proliferate. More potent concentrations of peroxide, although more destructive, were observed to precipitate and modify the whey proteins.

The bactericidal limitations of a 0.02% final concentration of H_2O_2 on milk-souring types of organisms were investigated. Within 1 hr., a 97% kill of 2.8×10^7 bacteria was effected, a remarkable achievement considering that this number of bacteria represents a grossly contaminated material. Studies showing 100% destruction of milk microorganisms have been reported by Nambudripad (6), who found 16 hr. of exposure to 0.05% H_2O_2 at 37° C. necessary for full destruction of *Bacillus megatherium* in broth solutions. This contrasted greatly to the much shorter time of 30, 40, and 45 min. required for 100% killing of *Aerobacter aerogenes, Alcaligenes viscosus,* and *Escherichia coli,* respectively, all Gram-negative and peroxide-sensitive forms.

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